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TITLE: Development of an Advanced Injectable Therapy for Ischemic Vascular Disease

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14. ABSTRACT
Cardiovascular diseases are the most common causes of death for Americans and it is estimated that 20% of the population over 65 years of age have peripheral arterial disease. The clinical standard of care for chronic peripheral ischemia includes physical therapy, pharmacological interventions, endovascular stent placement and surgical bypass of stenosed arteries. While these methods can often restore perfusion to the ischemia limb, these therapies often fail in the long term due to continued microvascular disease. In addition, many patients are not unable to be treated with these therapies due to overall poor health or diffuse vascular disease. A promising approach to this problem is to induce the growth of new vessels using angiogenic therapy with growth factors to restore flow to the ischemic tissues. Unfortunately, many clinical trials using growth factors for treating ischemia have shown little benefit for patients. Our group has recently identified that the long-term disease processes in diabetes and hyperlipidemia reduce the levels of the growth factor co-receptor syndecan-4 in human patients. Loss of this co-receptor would prevent growth factor therapy from being effective in these patients.

15. SUBJECT TERMS

None provided

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1. Introduction

Cardiovascular disease is a prominent cause of death worldwide with an estimated 17 million deaths each year. A key contributor to this disease is atherosclerosis, or the thickening of arterial walls through lipid accumulation. This condition leads to a reduction of blood flow and the creation of local ischemia. Left untreated, ischemia can lead to major organ dysfunction and failure. Currently, atherosclerosis is treated with either percutaneous interventions to physically expand or prop open the artery or surgery to reroute blood flow to the poorly perfused tissue. Percutaneous interventions such as angioplasty and stenting have major limitation for long term treatment of ischemia while bypass surgeries are strongly associated with significant rate of morbidity and mortality. Our approach aims to grow new arteries and microvasculature from the existing vessels to perfuse ischemic areas in an effective and physiological manner. Our system involves the delivery of both the proteoglycan syndecan-4 as well as growth factors to treat ischemia in growth factor resistant disease states such as diabetes. To achieve this, we will undertake a large animal preclinical trial to reproduce the positive results that we have seen in smaller animal model angiogenesis and wound healing.

2. Keywords

Atherosclerosis, diabetes, ischemia, syndecan-4, peripheral vascular disease, proteoliposomes.

3. Accomplishments

Major Goals of the Project

The overall purpose of this study is to create a new option for the treatment of cardiovascular disease and atherosclerosis: one that is both more efficacious in the long-term outlook of patient health and one that mitigates the risk of mortality during surgery. Utilizing syndecan-4 proteoliposomes, this project aims to produce a viable therapy and acquire enough data to file an Investigational New Drug (IND) application for entering clinical trials.

The required tasks to complete this project are reflected through the following tasks:

Task 1. Optimize the formulation of a controlled release gel that is injectable and delivers proteoliposomes with tailorable release kinetics as a local therapeutic for ischemia.

Task 2. Evaluate the efficacy of syndecan-4 proteoliposomes delivered from an injectable gel in a preclinical rabbit model of hind limb ischemia in hyperlipidemic rabbits.

Task 3. Perform manufacturing scale up and detailed biocompatibility and toxicology studies on the optimized therapeutic formulation and treatment regimen.

In the first year of this project, the following work and goals were planned.

Aim 1. Optimize the formulation of a controlled release gel that is injectable and delivers proteoliposomes with tailorable release kinetics as a local therapeutic for ischemia.		
	Timeline	**Finished Date**
Task 1.1. Develop a set of injectable alginate formations for the slow and fast release of syndecan-4 proteoliposomes (Lead Investigator: Baker).	Months	
Subtask 1.1.1. *In vitro* release kinetics of alginate formulations.	1-3	6/30/2017
Subtask 1.1.2. Cryo-EM, dynamic light scattering and tube formation assay.	1-3	6/30/2017
Milestone(s) Achieved:		
M1. Establish *in vitro* release profiles	3	6/30/2017
M2. Determine if syndecan-4 proteoliposomes are released intact and active from injectable gel	3	6/30/2017
Task 1.2. Toxicity studies with FGF-2 and syndecan-4 proteoliposomes (Lead Investigator: Baker).	Months	
Subtask 1.2.1. Study of acute toxicity in mice	4	In progress (25%)
Subtask 1.2.2. Histological analysis of tissues	5-6	In progress (25%)
Milestone(s) Achieved:		
M3. Determination of toxicity of FGF-2 and syndecan-4 proteoliposomes.	6	In progress (25%)
Task 1.3. Pharmacokinetics/Pharmacodynamics (PK/PD) of locally delivered S4PL in diabetic mice (Lead Investigator: Baker).	Months	
Subtask 1.2.1. Pharmacokinetic study with radiolabeled FGF-2 and syndecan-4.	6-9	In progress (25%)
Subtask 1.2.2. Histological analysis of tissues and cryosectioning for tissue distribution	9-12	In progress (25%)
Milestone(s) Achieved:		
M4. Determination of pharmacokinetics of FGF-2 and syndecan-4 proteoliposomes.	12	In progress (25%)

Aim 2. Evaluate the efficacy of syndecan-4 proteoliposomes delivered from an injectable gel in a preclinical rabbit model of hind limb ischemia in hyperlipidemic rabbits		
	Timeline	**Finished Date**
Task 2.1. Preliminary and Baseline studies on diabetic, hyperlipidemic rabbit model (Lead Investigator: Smalling).	Months	
Subtask 2.1.1. Preliminary and baseline tests of with non-treated animals.	1-7	9/30/2017
Subtask 2.1.2. Baseline test on rabbits treated with alginate.	7-12	In progress (30%)
Milestone(s) Achieved:		
M5. Validate hind limb ischemia model in diabetic hyperlipidemic rabbits.	7	9/30/2017
M6. Establish baseline ischemia recovery for non-treated and alginate treated rabbits.	12	In progress (30%)

What was accomplished under these goals?

During the first year of the project, we have successfully optimized an effective method of alginate injection, quantified release profiles from this method using growth factor release, fully validated the hind limb ischemia model in rabbits, and begun the tests for the establishment of baseline recovery for both non-treated and alginate treated rabbits. The animal model optimization and validation required considerably more time than anticipated due to issues with the animals dying before the end of the study. This was due to complications involving diabetic induction or death following the surgical procedure in the diabetic animals. We worked to optimize the model by lowering the cholesterol/fat content in the animal chow. In addition, we altered the protocol for inducing diabetes to slow the infusion of the drug to induce diabetes. This aided in the effective establishment of diabetes without other complications.

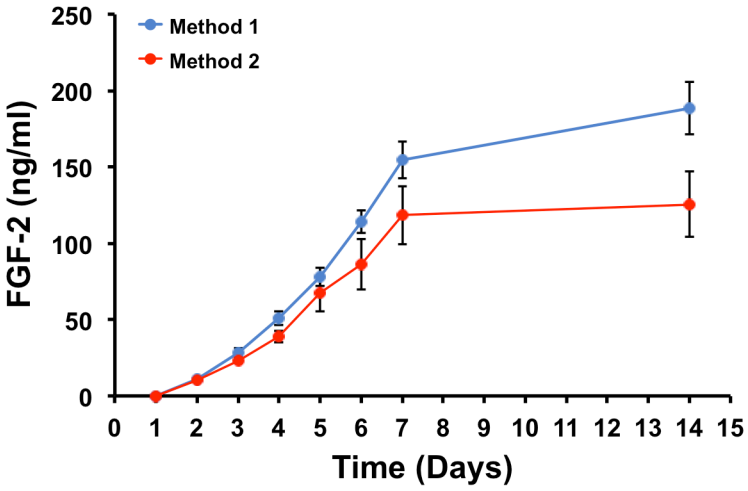

Figure 1. FGF release profile over 14 days. FGF was suspended in alginate gel and crosslinked in calcium sulfate. Gels were placed in saline in a shaker at 37°C for the span of the study.

Our initial tests of the gel injection protocol determined that further optimization of the delivery gel and injection protocol were needed. We performed studies using two potential injection systems and tested the number/volume of injections to optimize these to deliver a significant volume of gel without side effects. We tested both numerous combinations of alginate gel and chemical crosslinker as well as injection methods to find the optimal injection technique. We consolidated the methods to two techniques. Method 1 involved the use of two syringes connected with a y-linker; one syringe carried 2% sodium alginate while the other held the crosslinking agent, calcium sulfate, at 0.01 g/ml. Method 2 used one syringe which housed both the 2% alginate and calcium sulfate at a lower concentration of 0.002 g/ml. Release studies were conducted using the growth facor FGF for both gels (**Figure 1**). Similar release profiles were

detected for each method. Because of its simplicity compared to method 1 and similar release, method 2 was chosen.

The rabbit hind limb ischemia surgery involves the ligation and removal of the right femoral artery, creating an area of local ischemia (**Figure 2A, B**). Angiograms were performed before and after the ligation and removal of the artery to show the archetechture of the vasculature (**Figure 2C**). The femoral artery is easily detectable before surgery and notably missing in the angiogram post-surgery. Forty-five days after surgery, an additional angiogram was performed to assess recovery of vasculature in the ischemic area (**Figure 3**). Angiograms have been collected for both non-treated and alginate treated rabbits to establish a baseline of recovery.

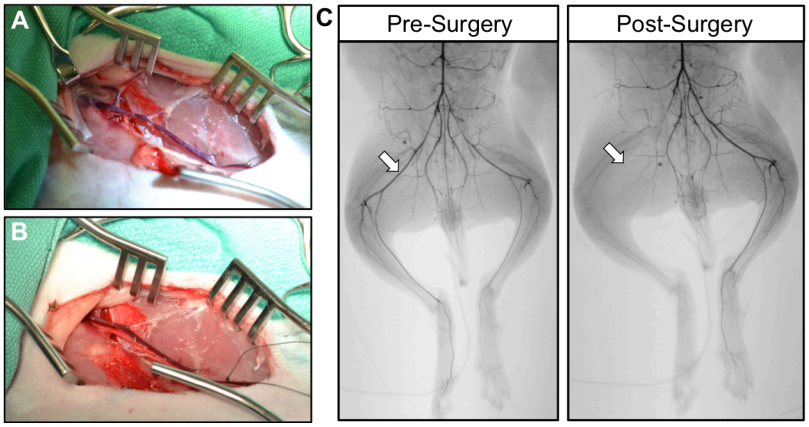

Figure 2. Hind limb ischemia surgery in diabetic rabbits. (A) Isolation of the femoral arteries in the diabetic rabbit. (B) Femoral arteries after multiple ligations. (C) Digital angiographs from rabbits pre- and post-hind limb ischemia surgery.

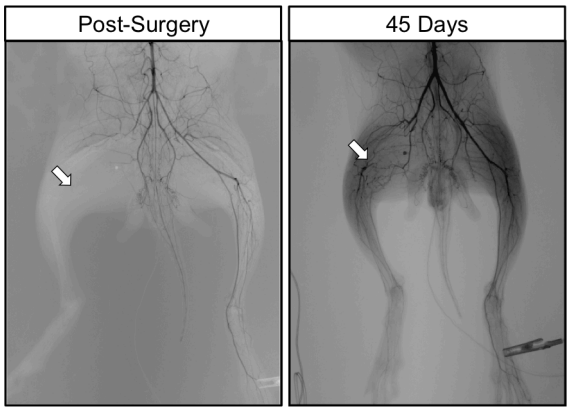

Figure 3. Angiogram of alginate treated rabbit showing baseline recovery of vasculature in the ischemic area after 45 days.

Training and professional development provided by the project

Graduate students and post-doctoral students have been trained on the scale-up and manufacturing components of proteoliposome creation. Graduate students and postdoctoral fellows have attended conferences related to biomedical engineering and vascular biology to present their work and learn about the work of other laboratories examining therapies for ischemia.

How were the results disseminated to communities of interest?

Nothing to report.

What do you plan to do during the next reporting period to accomplish the goals?

Over the next 12 months we will perform the baseline studies in rabbits and begin performing the test studies of the therapies in the animals. We plan to make up some of the delayed time by increasing the number of rabbits in the experiment at one time, within the limits of the animal facility resources and staff time available. During the baseline studies, we will perform the toxicology and pharmacokinetics studies so that there will not be a delay in beginning with the optimized treatment studies.

4. Impact

What was the impact on the development of the principal discipline(s) of the project?

The development of the diabetic, hyperlipidemic, rabbit hind limb ischemia model provides a model of ischemia recovery in a large animal setting. This enhanced preclinical model may be more predictive of the response of humans to interventions for peripheral vascular disease and can consequently aid in preclinical studies for drugs and surgical procedures for this indication. Our lab intends to increase the visibility of this model through a publication in the *Journal of Visualized Experiments*.

What was the impact on other disciplines?

Other disciplines may benefit from a model of diabetes in rabbits that is as severe as possible without compromising the survivability of the animals. Therapies such as glucose sensors, glucose pumps, artificial pancreas and tissue engineered vascular grafts may benefit from being tested in the advanced preclinical model created in our work.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

None to report.

5. Changes/Problems

Changes in approach and reasons for change

None to report.

Actual or anticipated problems or delays and actions or plans to resolve them

There were several delays in the overall progress due to the initial formulation of the gel not working optimally and the need to develop a technique for injection into rabbits. Optimization experiments were done to ensure that alginate could be easily injected into the muscle of the ischemic leg without premature crosslinking in the syringe. Various combinations of alginate and crosslinking agents were used to find the correct components. Furthermore, many different syringe systems were tested before being narrowed down to two methods that were tested for growth factor delivery. The optimal method was also tested in tissue where it properly crosslinked and embedded within the muscle. This process took time away from out completion of Milestones 2 to 4.

The diabetic, hyperlipidic, rabbit model proved difficult to implement as rabbit blood glucose levels would vary unpredictably after induction. Even with dextrose and insulin treatments, rabbit blood glucose levels would remain at extreme values with little modulation from the treatment. This led to a number of rabbit deaths both after induction and after surgery. To solve this issue, we increased the duration of the diabetic induction with alloxan from 4 minutes to 15 minutes. We have also lowered the cholesterol in the rabbit feed to improve their condition after induction and surgery. These difficulties resulted in significant delays in the development of the rabbit model but have been corrected and are no longer an issue.

Changes that had a significant impact on expenditures

None to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

None to report.

Significant changes in use or care of human subjects

None to report.

Significant changes in use or care of vertebrate animals.

None to report.

Significant changes in use of biohazards and/or select agents

None to report.

6. Products

Publications, conference papers, and presentations

None to report.

Website(s) or other Internet site(s)

None to report.

Technologies or techniques

None to report.

Inventions, patent applications, and/or licenses

None to report.

Other Products

None to report.

7. Participants & Other Collaborating Organizations

What individuals have worked on the project?

Name:	*Aaron Baker, PhD*
Project Role:	*Principal Investigator*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	3
Contribution to Project:	*Investigator responsible for oversight of the study*
Funding Support:	3 months

Name:	*Richard Smalling, MD, PhD*
Project Role:	*Interventional Cardiologist*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	0.6
Contribution to Project:	*Collaborating researcher responsible for oversight of the study; performs surgical procedures/angiograms.*
Funding Support:	0.6 months

Name:	*Julia Goldman, DVM*
Project Role:	*Clinical Veterinarian*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6
Contribution to Project:	*Provides veterinary care and oversight; currently training to perform surgical procedures/angiograms.*
Funding Support:	6 months

Name:	*Gretchen Howe, LVT, RLAT*
Project Role:	*Senior Research Associate*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	2.28
Contribution to Project:	*Prepares cholesterol chow and Alloxan; administers Alloxan injections and provides post-injection monitoring; provides surgical assistance; runs fluoroscope; conducts tissue harvest.*
Funding Support:	2.28 months

Name	*Patricia Felli*
Project Role:	*Research Associate*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	4.8
Contribution to Project:	*Provides surgical assistance; runs fluoroscope.*
Funding Support:	4.8 months

Name	*Andrew Sligar*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6
Contribution to Project:	*Andrew has worked with drug release studies and alginate preparation.*
Funding Support:	6 months

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

None to report.

What other organizations were involved as partners?

Subcontract with UT Health Sciences (Houston, TX).

8. Special Reporting Requirements

None to report.

9. Appendices

None to report.